



RESEARCH ARTICLE

The Effect of the Major Components of Fructus Cnidii on Osteoblasts *In Vitro*

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Abstract

In traditional Chinese medicine, the cause of weak bones or bone loss is generally regarded as a result of kidney deficiency. Fructus Cnidii (FC), which is also known as She-Chuang-Zi, is a traditional herb that has been claimed to have kidney warming effects that invigorate Yang. In this study, we tried to determine the bone production-inducing effect of FC on osteoblastic cells *in vitro* using osthole, the main component of FC. Osteoblasts were isolated from neonatal Sprague-Dawley rat calvaria using the tissue piece culture method and treated with various concentrations of osthole ranging from 2.5 to 640 µg/mL, together with a blank control. Cell proliferation, alkaline phosphatase (ALP) activity, and bone nodules were measured. The cells were examined by hematoxylin-eosin staining, the Gomori Calcium-Cobalt method and immunofluorescent staining. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (or MTT) assay, ALP assay, and bone nodule results indicated significantly enhanced osteoblastic proliferation and differentiation at concentrations of osthole ranging from 40 to 320 µg/mL. Concentrations lower than 40 µg/mL seemed less effective, and cytotoxicity to osteoblasts was observed at concentrations higher than 320 µg/mL. These results indicate that osthole is effective at inducing osteoblastic bone formation through the up-regulation of ALP activity. FC is a Chinese herb used to treat lumbar pain in clinical practice. Further studies concerning the effects and mechanism of osthole on osteoporotic patients and animals should be performed, as these studies may lead to the development of a drug treatment for osteoporosis in the future.

1. Introduction

Bone homeostasis requires balanced interactions between osteoblasts and osteoclasts. Osteoporosis, therefore, involves a reduction in skeletal mass due to an imbalance between bone resorption and bone formation [1]. Over the past 10 years, patients with osteoporosis have been treated with antiresorptive agents (estrogens, bisphosphonates, calcitonin), which reduce osteoclast bone resorption. These

agents prevent bone from being broken down; allowing remodeling spaces to be filled, and improve bone strength and reduce fracture risk [2]. Recently, attention has turned away from osteoclast inhibition and towards agents including parathyroid hormone, growth hormone, insulin-like growth factor-1, bone morphogenetic protein-2, and vascular endothelial growth factor, which stimulate osteoblasts to form new bone and anabolic agents [3]. Therefore, anabolic agents with the ability to stimulate

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new bone formation and correct imbalances of trabecular microarchitecture, which are characteristic of established osteoporosis, are desirable [4]. Among these, *in vitro* studies, especially studies related to components isolated from Chinese herbal medicine, are gaining attention [5].

Osthole is one of the main components of the dried seeds from the *Fructus Cnidii* (FC) plant, which is also known by its traditional name *She-Chuang-Zi* (SCZ) [6]. FC is known for its effects including kidney warming, which is thought to invigorate Yang, and the clearance of heat and toxic materials from the body. FC is used in Traditional Chinese Medicine (TCM) in tonics and aphrodisiacs to treat impotence and lumbar pain in clinical practice [7,8]. Weak bones and bone loss are generally regarded to be caused by kidney deficiencies in TCM, but the bone-forming actions of FC on osteoporosis have rarely been reported. Osthole was reported to exhibit estrogen-like effects, preventing postmenopausal osteoporosis in ovariectomized rats [9], however, the biological effects of osthole on bone cells are relatively unknown. In this study, we determined the bone-inducing effects of osthole, the main component of FC, on osteoblast cells *in vitro*. We also describe the promoting activities of different concentrations of osthole on osteoblast cells.

2. Materials and Methods

2.1. Reagents and materials

The Chinese herb FC was obtained and its component osthole (7-methoxy-8-isopentenoxycoumarin, molecular weight: 244.29) was prepared by the National Institute for the Control of Pharmaceutical and Biological Products (product number: 110822-200406). Nine pre-experimentally-determined concentrations of osthole (0, 2.5, 5, 10, 20, 40, 80, 160, 320, and 640 $\mu\text{g/mL}$) were diluted with DMEM-F12 (Invitrogen, Carlsbad, CA, USA) containing 15% fetal bovine serum (FBS; Hyclone, Thermo Scientific, Waltham, MA, USA) and 1% penicillin-streptomycin. All solutions used in cell cultures were sterilized by filtration through 0.2 μm Millipore filters and stored in a refrigerator at 4°C until use. D-Hanks' solution, penicillin G, streptomycin, and all other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan), unless stated otherwise.

2.2. Cell culture

Primary osteoblastic cells were prepared from neonatal (0–3 days old) Sprague-Dawley rats (CLEA Japan, Osaka, Japan). The rats were housed in a constant temperature-controlled environment (23°C)

with 60% relative humidity under a 12-hour light/dark cycle. The procedures involving the animals and their care were in full compliance with our institutional regulations and the current international laws and policies [10]. The rats were killed by being immersed in 70% alcohol for 10 minutes, and then the rat calvaria were excised, stripped of soft tissue, washed three times in D-Hanks' solution containing 0.25% trypsin, and cut into small pieces with a dissector at a volume of about 1 mm^3 . The bone tissue pieces were homogeneously seeded at 1 cm intervals in flasks, which had been incubated in advance for 10 minutes with 1 mL of DMEM medium. After being kept inversely in an incubator at 37°C in a 5% CO_2 fully humidified atmosphere for 2 hours, the flasks were then reversed to their normal position and 5 mL of DMEM/F12 containing 15% FBS and 1% penicillin-streptomycin was added in order to avoid adhered tissue pieces floating to the surface from the bottom. Flasks were then incubated continuously for 3 days in an incubator. Media was replaced every 3 days. After reaching confluence, the cells were passaged every 7 days.

2.3. Identification of osteoblast cells

Cellular morphology and growth were observed daily, after the first 3 days in primary culture, using an inverted microscope, whereupon images were obtained. We used methods similar to those described elsewhere for the identification of osteoblasts, in addition to hematoxylin-eosin staining [11]. Alkaline phosphatase (ALP) staining was also performed using the Gomori Calcium-Cobalt (Ca-Co) method. In brief, when the cells had reached confluence, after fixation with a solution of 95% alcohol for 10 minutes, the cells were incubated in an incubator at 37°C for 4–6 hours. The cells were then stained with solutions of 2% cobalt nitrate and 1% ammonium sulfide in turn. After being air-dried, the slides were finally mounted and used for microscopy [12]. Immunocytochemistry, as described by others, was also performed to identify osteoblasts [13]. Briefly, cells were planted onto a coverslip. When 70% confluence had been reached, the coverslips were fixed using a 95% alcohol solution for 5 minutes. Mouse anti-collagen (type clone Col-1: 2456) was used as a primary antibody at a ratio 1:100, which was diluted in 0.3% Triton X-100 and 5% normal goat serum in phosphate buffered saline (PBS). The cells were incubated with the primary antibody present at 4°C overnight before being washed in PBS. Anti-mouse IgG TRITC antibody produced in goats (Sigma-Aldrich, St-Louis, MO, USA) was used as the secondary reagent (1:100) after being diluted in 0.3% Triton X-100/PBS and incubated for 2 hours at 4°C. After a final

brief wash, the coverslips were mounted in glycerol and viewed with a fluorescent microscope.

2.4. Colorimetric assay for cell viability

An [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] (MTT) assay was performed to estimate cell proliferation, according to the method described by Singh et al [5]. The concentrations of osthole used were determined according to the changes in growth curves, which reflected the correlation between the various concentrations and cell numbers in our pre-examination (data not shown). Negative controls were treated with the same supplemented medium but without the presence of drugs. When about 80% confluence had been reached, the cell layer was digested by trypsin and cultured in a 96-well culture plate (5×10^3 cells/well) for 24 hours. The culture medium was replaced with solutions of varying concentrations of osthole (0, 2.5, 5, 10, 20, 40, 80, 160, 320, and 640 $\mu\text{g/mL}$) and incubated for 24 hours. MTT was dissolved in PBS at a concentration of 5.0 mg/mL. MTT solution (50 μL) was added to each well of a culture plate containing 200 μL medium and incubated at 37°C for 4 hours. The supernatant was then carefully removed without disturbing the dark blue formazan crystals. DMSO (50 μL) was then added to each well and mixed thoroughly to dissolve the formazan crystals. The plates were then read using a multiskan plate reader (Multiskan JX; Labsystems, Tokyo, Japan) with a wavelength of 540 nm. Readings are presented as optical density values.

2.5. Analysis of ALP

After 7 days of culture, the differentiation of osteoblasts was determined by measuring their ALP activity. The medium was removed from the wells, and the cell layer was washed twice with PBS. 0.1% Triton X-100 (Sigma-Aldrich; 100 μL /well) was used to lyse the cells for 5 minutes at room temperature. The chromogenic substrate used to measure ALP was 10 mM 4-nitrophenyl phosphate (Amresco, Solon, OH, USA). The ALP concentration was determined by kinetic measurement of the absorbance at 405 nm using standards with defined ALP concentrations [14].

2.6. Bone nodules

Von Kossa's stain was utilized to assess the formation of the mineralized matrix. A total of 2×10^4 osteoblast cells/wells were added to culture medium containing 1% FBS, 1% penicillin-streptomycin, 50 $\mu\text{g/mL}$ ascorbic acid, and 10 mM β -glycerophosphate;

the cells were incubated for 21 days in a 24-well plate. Culture medium was mixed with one of the nine concentrations of osthole used in the MTT and ALP assays at a volume ratio of 9:1. The medium was replaced every 2 days. After 21 days, the cultured cells were fixed with a 100% methanol solution and stained with the Alizarin Red Method [15]. Mineralization was quantified by visual counting at high magnification of five random fields with an optical microscope. Orange-red nodules with sharp borders and a diameter larger than 200 μm were counted, and the mean value was taken as the final result [16].

2.7. Statistical analysis

Data are expressed as mean \pm SEM. Statistical differences among groups were evaluated by one-way analysis of variance using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). The statistical differences between the treatment and control were determined by the Fisher's least significant difference test. The levels of statistical significance were set to $p < 0.05$, and the results that met this condition are indicated in the figures with an asterisk.

3. Results

3.1. Observation of cell morphology

The numbers of cultured cells gradually began to increase after 3 days of incubation of the tissue pieces. Cells were seen to adhere to the bottom of the culture dish and possessed typical shapes such as triangular, fusiform, and polygonal, with one or two elliptical nuclei in the center of the cells (Figure 1A). Following this observation, the cell numbers increased gradually and reached confluence at 10 days of incubation. Figure 1B shows osteoblast cells of the second passage at 5 days, from which projections had formed, with the cells appearing to grow in a radial fashion. Hematoxylin-eosin staining images showed high purity of cells and the same characteristics as described above. Nuclei were stained an amethyst color, and the cytoplasm was stained pink (Figure 1C). To confirm that the cultured cells were osteoblasts, in addition to the hematoxylin-eosin staining described above, Ca-Co and immunofluorescence staining were applied to identify the osteoblastic cells. Cellular cytoplasm was stained grayish-black due to ALP staining and a brown-stained positive reaction was observed together with yellow shiny nodules marked by tetracycline. These findings are consistent with other studies [10,13] characterizing osteoblast histology (Figure 1D).

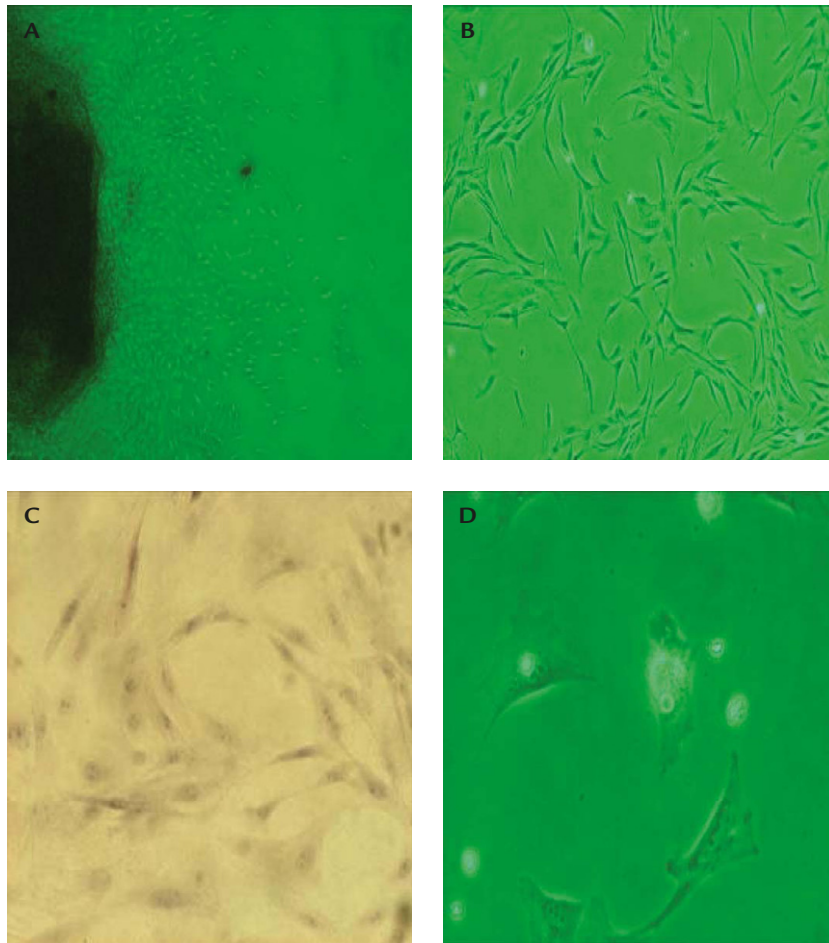


Figure 1 (A) Primary cultured osteoblast cells at 3 days (4 \times). (B) Osteoblast cells of the second passage at 5 days (40 \times). (C) Hematoxylin-eosin staining (100 \times). (D) Immunofluorescence staining (200 \times).

3.2. Effects of osthole concentration on osteoblast activity

The effects of various concentrations of osthole on the proliferation of osteoblast cells was measured by an MTT assay according to the method described above (Figure 2). Osthole concentrations lower than 10 $\mu\text{g/mL}$ did not affect the proliferation of osteoblasts. However, osthole was effective at concentrations higher than 20 $\mu\text{g/mL}$, and significant enhancement was seen at concentrations from 40 to 320 $\mu\text{g/mL}$ compared with the blank control ($p < 0.05$). The most effective concentration was 160 $\mu\text{g/mL}$. However, the proliferative activity of osthole dropped at concentrations higher than 320 $\mu\text{g/mL}$.

Figure 3 shows the effects of various concentrations of osthole on the ALP activity of osteoblasts. Osthole concentrations lower than 40 $\mu\text{g/mL}$ produced less ALP upregulation. Additionally, increases in concentration dependent cell differentiation became more evident and were significantly enhanced at concentrations ranging from 40 $\mu\text{g/mL}$ to 320 $\mu\text{g/mL}$ compared with the blank control group ($p < 0.05$). The most effective action was seen at 80 $\mu\text{g/mL}$.

As for MTT, concentrations of osthole higher than 320 $\mu\text{g/mL}$ inhibited the total metabolic activity of osteoblasts.

3.3. Observation of bone nodules

Figure 4 shows the number of total calcified nodules formed in osteoblast cultures at various osthole concentrations. As a matter of principle, the nodule number should correlate with the effects of MTT and ALP, which reflect the proliferation and the activities of bone cells. However, in the presence of various concentrations of osthole treatment, significant changes in the number of nodules were only seen at 40 and 80 $\mu\text{g/mL}$.

4. Discussion

The enzymatic digestion method is most commonly used to culture osteoblast cells *in vitro* and is considered to have the important advantage of easily obtaining large quantities of cells. This method also has disadvantages, such as a lower degree of cell

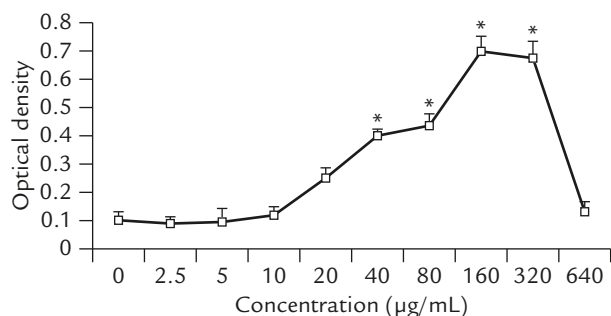


Figure 2 Effects of osthole at various concentrations on the proliferation of osteoblast cells *in vitro*, as determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide or MTT assay (* $p < 0.05$ vs. control).

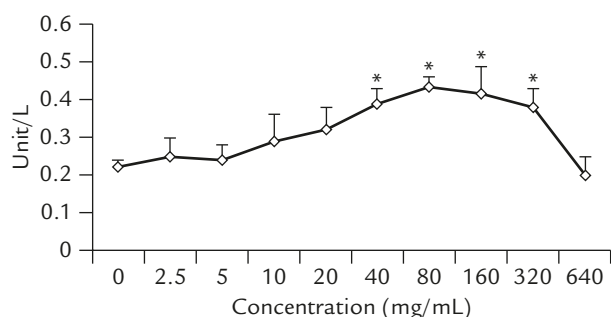


Figure 3 Effects of osthole at various concentrations on the differentiation of osteoblasts *in vitro*, as determined by an alkaline phosphatase (ALP) activity assay (* $p < 0.05$ vs. control).

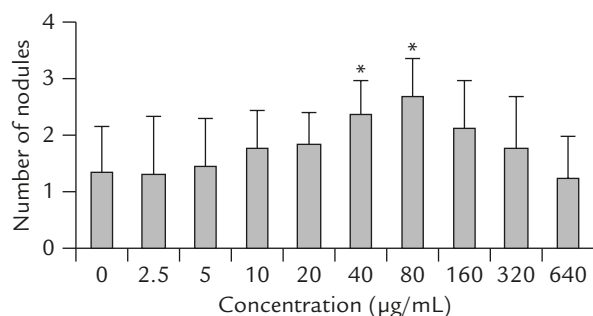


Figure 4 Effects of osthole at various concentrations on the formation of mineralized nodules in an osteoblast culture system (* $p < 0.05$ vs. control).

purity due to the complicated manipulation process and the possibility of affecting the viability and adherence of cells due to the enzymatic process involved. Using bone tissue pieces also has its difficulties, such as obtaining a sufficient quantity of cells and a lower speed of cell liberation [17]. In this study, we modified the traditional tissue piece method by incubating the flask for 1 hour in advance with DMEM/F12 medium at 37°C in an incubator. This produces better viability and a larger quantity of osteoblasts than the methods that lacked pre-incubation that we tested in our pilot study

(data not shown). Moreover, cell identification by hematoxylin-eosin, ALP specific staining, and immunofluorescence staining also confirmed a higher purity of osteoblasts; therefore, we suggest that pre-incubating is a very important process for the tissue piece method.

Several Chinese herbs and herb extracts have proven effective at improving bone properties and enhancing mineralization *in vitro* and have thus been shown to be potential bone-inducing agents [15,18]. Since there is a close relationship between kidney and bone production, a majority of these studies have focused on medicines with kidney-tonifying activities, such as *Fructus Ligustri Lucidi* (Nv-Zhen-Zi), *Hominis Placenta* (Zi-Hec-Che), *Herba Epimedii* (Yin-Yang-Huo) and *Rhizoma Drynariae* (Gu-Sui-Bu). FC is a medicine that is used in TCM to warm the kidney as a way of invigorating Yang, expel cold and wind, eliminate dampness and destroy parasites. Thus, FC is used to treat impotence, sterility due to uterine coldness, leucorrhea due to cold dampness, lumbago due to damp arthralgia, pudendal damp itching, and eczema. Kuo et al [19] reported osthole-mediated cell differentiation through the bone morphogenetic protein-2/p38 and extracellular signal-regulated kinase 1/2 pathway in human osteoblast cells; whereas, no significant increase in the proliferation of MG-63 or hFob cells was exhibited.

We observed the bone-inducing activities of osthole using MTT and ALP assays to examine the proliferation and differentiation of osteoblasts. The addition of osthole at concentrations lower than 40 µg/mL did not affect the proliferation or differentiation of osteoblasts. ALP is a protein involved in bone metabolism and one of the marker enzymes of mature osteoblastic cells, which plays an important role during calcification *in vitro*. Its level commonly increases in the early period of osteoblastic differentiation and decreases in the mineralized period [13]. In our study, concentrations ranging from 40–320 µg/mL caused corresponding increases in total metabolic activity and ALP activity in a dose-dependent manner, which indicates that osthole induces osteoblastic bone formation through the upregulation of ALP activity, however, a concentration of 640 µg/mL did inhibit the growth of osteoblast cells and produced cytotoxic activity.

By contrast, the positive staining of mineralized bone nodules by Von Kossa's stain, which was attributed to increased cell viability, not only reflects increased bone deposition but also that the osteoblasts had matured. Concentrations from 40–160 µg/mL tended to increase the formation of bone nodules compared with the blank control group, but significant increases were only detected at 40 and 80 µg/mL. Previous studies reported different

observation periods such as 14 days, or longer than 25 days [12,17]. We suppose that the reduced effects on the mineralization of nodules seen at concentrations from 160–320 µg/mL may be attributed to our longer follow-up period of 21 days, which led to the possibility of a higher density of osteoblasts forming due to their active proliferation. Also, the presented congregation and overlapping limited the further growth of osteoblast cells. Additionally, the inconsistency between the results for the bone nodules and the ALP data also confirmed that osteoblastic differentiation decreased in the mineralized period.

Based on the above findings, this study demonstrates that osthole not only considerably stimulated osteoblastic proliferation and differentiation, but also enhanced the deposition of calcium and phosphate at concentrations ranging from 40–160 µg/mL. Other studies have reported that osthole has an estrogen-like effect and prevents postmenopausal osteoporosis in ovariectomized rats [9]. To investigate whether the bone-inducing actions seen in this *in vitro* study can also be attributed to the same effect, an additional experiment involving co-incubation with an estrogen receptor antagonist should be performed to determine whether the effects of osthole on cell proliferation, differentiation, and mineralization are estrogen receptor dependent.

In conclusion, this study demonstrated that osthole accelerates osteoblastic proliferation and differentiation at concentrations from 40–160 µg/mL and that concentrations near to 80 µg/mL seem to be the most effective experimental concentration for inducing bone nodule formation. These findings support the idea that osthole is effective at inducing osteoblastic bone formation through the upregulation of ALP activity, and since findings from cell culture and animal experiments concur, the development of a pharmaceutical therapy for osteoporosis may be possible in the future.

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